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# Branched-chain amino acids influence the immune properties of microglial cells and their responsiveness to pro-inflammatory signals

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ABSTRACT

The branched-chain amino acids (BCAAs) valine, leucine and isoleucine are essential amino acids involved in several important brain functions. Although commonly used as nutritional supplements, excessive intake of BCAAs might favour the establishment of neurotoxic conditions as indicated by the severe neurological symptoms characterising inherited disorders of BCAA catabolism such as maple syrup urine disease (MSUD). Recent evidence indicates that BCAAs induce excitotoxicity through mechanisms that require the presence of astrocytes. In the present study, we evaluated the effects of BCAAs on microglia, the main immune cells of the brain. As an experimental model we used primary microglial cells harvested from mixed glial cultures that had been kept in normal or high BCAA medium (H-BCAA). We show that H-BCAA microglial cells exhibit a peculiar phenotype characterized by a partial skewing toward the M2 state, with enhanced IL-10 expression and phagocytic activity but also increased free radical generation and decreased neuroprotective functions. We suggest that such an intermediate M1/M2 phenotype might result in a less efficient microglial response, which would promote the establishment of a low grade chronic inflammation and increase the likelihood of neurodegeneration. Although based on in vitro evidence, our study adds on to an increasing literature indicating that the increasing use of dietary integrators might deserve consideration for the possible drawbacks. In addition to excitotoxicity, the altered immune profile of microglia might represent a further mechanism by which BCAAs might turn into toxicants and facilitate neurodegeneration.



## 1. Introduction

Valine, leucine and isoleucine are essential amino acids that share side chain structure features to which the collective term branched-chain amino acids (BCAAs) refers to. Unlike most of amino acids, only a minor fraction of the dietary BCAAs is metabolized by the liver; the largest part of BCAAs enters the systemic circulation to reach the main sites of BCAA metabolism, namely skeletal muscles, adipose tissue and brain [1,2].

It has been estimated that in healthy subjects about 10% of infused BCAAs reach the brain where they participate to several important biochemical processes such as protein synthesis, energy production and glutamate synthesis [1–3].

Although commonly used as nutritional supplements to improve mental and physical performance [4,5], excessive intake of BCAAs might favour the establishment of neurotoxic conditions and exert negative consequences on brain functions. Neurotoxic effects of BCAAs or their catabolic products are supported by the severe neurological symptoms characterising inherited disorders of BCAA catabolism such as maple syrup urine disease (MSUD), in which levels of BCAAs, as well as their branched-chain keto-acids, increases up to about 30 folds in blood, urine and cerebrospinal fluid, as compared to control subjects, [6–8]. The negative impact of high BCAA levels on neurons is further supported by recent experimental evidence indicating that BCAAs are neurotoxic per se and enhance excitotoxicity in cortical neuronal cultures through mechanisms that require the presence of astrocytes and involve NMDA receptor activation [9]. In addition, hyperexcitability of cortical neurons by BCAAs, which could facilitate excitotoxic events, has been demonstrated by electrophysiological recordings in both cultured neurons and in motor cortex slices from mice fed with BCAA-enriched diet [10].

*Abbreviations:* 15-F2t-IsoP, 15-$F_{2t}$-isoprostane; ADP, adenosine 5 -diphosphate; Arg-1, arginase-1; BCAAs, branched-chain amino acids; BME, basal Eagle's medium; IGF-1, insulin-like growth factor-1; IL, interleukin; iNOS, inducible nitric oxide synthase; KPBS, potassium-PBS; LPS, lipopolysaccharide; mMP, mitochondrial membrane potential; MRC-1, mannose receptor; MSUD, maple syrup urine disease; mTOR, mammalian target of rapamycin; NO, nitric oxide; PBS, phosphate-buffered saline; PFA, paraformaldehyde; TMRE, tetramethylrhodamine ethyl ester perchlorate; TNF-α, tumor necrosis factor-α; UDP, uridine 5 -diphosphate

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Besides neurons and astrocytes, high BCAA levels could influence the functional activities of other types of brain cells, including microglia, the main macrophage population of brain parenchyma. Microglial cells actively survey the brain parenchyma [11] to readily respond to signals released from damaged cells or pathogens. This physiological protective function, if not properly regulated, can turn into an aberrant microglial activation and contribute to neurotoxicity. As described for peripheral macrophages, the functions acquired by microglia during the process of activation can either promote or attenuate the inflammatory response, and though not yet fully defined, the repertoire of microglial phenotypes is likely to encompass the full spectrum ranging from the exemplified classically activated (pro- inflammatory, M1), to the alternatively activated (anti-inflammatory, M2) phenotypes [12]. The correct balance between pro- and anti-inflammatory activities is critical for preserving tissue homeostasis and several local cues can disrupt it, thus promoting neuronal impairment. BCAAs have been described to influence immune functions and in particular to be required for lymphocyte responsiveness and supporting of other immune cell functions [13] but, to the best of our knowledge, no studies have been devoted to brain immune response.

In the present study, we evaluated the effects of BCAAs on microglial reactivity through the analysis of several genes and markers associated with either M1 or M2 phenotypes. As an experimental model we used primary microglial cells harvested from mixed glial cultures that had been cultivated in normal or high BCAA medium (H-BCAA) for several days. We also compared specific functional properties of normal and H-BCAA microglial cultures, including migration, phagocytosis and reaction to a typical pro-inflammatory challenge such as bacterial endotoxin (lipopolysaccharide, LPS). We show that H-BCAAs influence microglial gene expression profile and immune properties, leading to an intermediate phenotype characterized by a partial skewing toward the M2 state, increased free radical generation and phagocytic activity, and altered responsiveness to LPS.

## 2. Materials and methods

### 2.1. Reagents

All cell culture reagents were from Invitrogen (Grand Island, NY, U.S.A) and virtually endotoxin free (less then 10E.U./ml as determined by the manufacturer). LPS (from Escherichia Coli, serotype 026:B6), ADP, UDP and BCAAs were obtained from Sigma (Saint Louis, MO, USA).

### 2.2. Cell cultures

Mixed primary glial cultures were obtained from the cerebral cortex of 1-day-old rats, as previously described [14] and in accordance with the European Communities Council Directive N. 86/609/EEC. Mixed primary glial cultures were maintained for 4 days in Basal Eagle's Medium (BME), supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine and 100 μg/ml gentamicin (control medium). Culture medium was then replaced by fresh control medium or high BCAA medium (H-BCAA), consisting in control medium supplemented with 10 mM valine, leucine and isoleucine (1:1:1) or additioned with 1 mM BCAAs when indicated. After 6 days, microglial cells were harvested by mild shaking and resuspended in control medium or H-BCAA and plated on uncoated plastic wells at a density of $1.5\times10^5$ cells/cm$^2$. Cells were allowed to adhere for 20 min and then washed to remove non-adhering cells. The cultures were maintained in control medium or H-BCAA for 24 h before further analysis and throughout the entire experimental period. Cell viability was greater than 95% in both culture conditions, as tested by Trypan Blue exclusion or MTT assay (see below).

### 2.3. Actin staining and immunocytochemistry

Purified microglia were plated on coverslips at a density of $1.5\times10^5$ cell/cm$^2$ and cultured for 24 h in control medium or 10 mM H-BCAA. Cells were then fixed with 4% PFA and pre-incubated in T-PBS (0.025% Triton in PBS) plus 5% BSA for 30 min, followed by incubation with rabbit anti-Ionized calcium binding adaptor molecule 1 (Iba1) antibody (Wako; 1:1000) in T-PBS plus 5% BSA for 1 h RT. After rinsing in T-PBS, cells were incubated with N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phallacidin (NBD-PhallAcidin, Molecular Probes; 100 U/ml, 1:30) to selectively label F-actin, and Cy3 donkey-anti-rabbit (Jackson Immunoresearch; 1:200) in PBS, 1 h at RT. Coverslips were then mounted with DAKO fluorescent mounting medium.

### 2.4. Reverse transcription and real time PCR

Total RNA (1 μg) from each sample was transcribed into cDNA using the RT-PCR Superscript III kit (Invitrogen, Eugene, OR, U.S.A.) according to the manufacturer's instructions. Real-time PCR was performed on the reverse transcription (RT) products with SensiMix SYBR Kit (Bioline, UK), or (for the arginase-1 mRNA expression) with SensiMix II probe Kit (Bioline, UK), following the manufacturer's instructions, using an ABI Prism 7000 Sequence Detection System (Applied Biosystems Inc, Foster City, CA, U.S.A.). Primer sequences (from MWG Operon, Ebersberg, Germany) and accession numbers are listed in Table 1. Annealing temperature was 60 °C for all the primer pairs listed. All samples were run in triplicate, and each well of PCR contained 20 μl as a final volume of reaction, including 2 μl of cDNA corresponding to 20 ng of total RNA, 750nM of each primer and 10 μl of PCR master mix. Thermal cycling conditions were as follow: 1 cycle at 95 °C for 10 min; 40 cycles 95 °C for 15 s and 60 °C for 1 min. Expression levels of genes of interest were compared between control unstimulated cultures and H-BCAA, LPS or LPS/H-BCAA cultures using the Relative Quantification (ΔΔCt) Study of Applied Biosystems 7000 System SDS Software. Hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as internal control gene and amplification specificity was checked using a melting curve, following the manufacturer's instructions. The reference group used is indicated in the Results section, for each study.

**Table 1**
Primers used for real time PCR analyses.

| Gene | Primers sequence | Acc. number |
|---|---|---|
| HPRT | Forward 5 -CTC ATG GAC TGA TTA TGG ACA GGA C-3<br>Reverse 5 -GCA GGT CAG CAA AGA ACT TAT AGC C-3<br>Product size:123 bp | S79292 |
| IL-1β | Forward 5 - CAC CTC TCA AGC AGA GCA CAG-3<br>Reverse 5 -GGG TTC CAT GGT GAA GTC AAC-3<br>Product size 79 bp | M98820 |
| iNOS | Forward 5 -GCC ACC TCG GAT ATC TCT TG-3<br>Reverse 5 -TCT GGG TCC TCT GGT CAA AC-3<br>Product size 81 bp | NM_0126113 |
| TNF-α | Forward 5 -AAAGGGCTCCCTCTCATCAGT-3<br>Reverse 5 -TCTGCTTGGTGGTTTGCTACGA-3<br>Product size 109 bp | NM_012675.3 |
| IL-10 | Forward 5 -GCCAAGCCTTGTCAGAAATGA-3<br>Reverse 5 -TTTCTGGGGCCATGGTTCTCT-3<br>Product size 73 bp | NM_012854.2 |
| Arg-1 | Forward 5 -ATATCTGCCAAGGACATCGTG-3<br>Reverse 5 -AGGTCTCTTCCATCACTTTGC-3<br>Probe: 5 -CAATGACTGAAGTGGACAAGCTGGGA-3<br>Product size 141 bp | N0_17134 |
| MRC-1 | Forward 5 -TGG ACT AAG CCA AGG GGC AA-3<br>Reverse 5 -CAG GAG CAG GGG GAG TCT CA-3<br>Product size 121 bp | NM_001106123 |

### 2.5. Migration assay

Migration of microglial cells was assessed using transwell migration chambers (8 μm-pore polycarbonate filters in 24-wells Costar, St. Louis, MO), according to previously published procedures [15]. In brief, the bottom wells were filled with medium alone or medium containing 10 μM ADP. Microglial cells were plated into the top wells ($6\times10^4$ cells/well) in control medium or H-BCAA, without serum, and incubated for 4 hours at 37 °C. Migrated cells were counted on the lower surface of the filters after fixation in 100% methanol for 3 min and hematoxylin and eosin (H&E) staining. Cells on the top side of the filter were wiped off using a cotton bud. Filters were then removed from the inserts and mounted on glass slides with the stained cells on the upper side, and the cells were counted under a light microscope (16 fields were examined for each condition).

### 2.6. Phagocytosis assay

Phagocytosis was evaluated by using 6 μm diameter fluorescent beads (Polysciences, Milan, Italy). The beads, purchased as aqueous suspension of 2.5% solid-latex (corresponding to $2\times10^8$ beads/ml), were opsonized for 30 min at 37 °C in Kreb's Ringer PBS containing 50% of fetal calf serum and then added to cells at the ratio of $10^5$ opsonized beads/$7\times10^4$ cells. Cells were incubated with beads at 37 °C in 5% $CO_2$ for 45 min. In some experiments, to induce the phagocytic activity of microglia, cells were pre-incubated with 100 μM UDP for 30 min at 37 °C, before performing the phagocytosis assay. After incubation, cells were washed twice with PBS for 2 min to remove excess beads. Cells were fixed with 4% PFA for 15 min at room temperature, washed for 10 min with PBS and subsequently processed for Iba-1 staining. The latex beads were visualized using fluorescence microscopy. The percentage of Iba-1 positive phagocytosing cells on total cell number in each field was counted. Ten fields for each experimental condition, run in triplicate, were counted in at least 3 independent experiments.

### 2.7. Cytokines, insulin growth factor-1, $F_2$-isoprostane and NO determination

Specific ELISAs for tumor necrosis factor-α (TNF-α, interleukin 1β (IL-1β), interleukin 10 (IL-10) (Endogen Inc., Woburn, U.S.A.) and insulin-like growth factor-1 (IGF-1, Boster Biological Technology, Rome, Italy) were used to assay microglial culture media, following the manufacturer's instructions. The ranges of determination were: 31–2500 pg/ml for TNF-α, 25–25,000 pg/ml for IL-1β; 16–500 pg/ml for IL-10; 31–2000 pg/ml for IGF-1.

Levels of 15-$F_{2t}$-isoprostane (15-$F_{2t}$-IsoP) were measured by a sensitive enzyme immunoassay (detection limit: 2 pg/ml; Cayman Chemical, Ann Arbor, MI, U.S.A.), according to the manufacturer instructions. All measurements were done at least in duplicate.

The production of nitric oxide (NO) was determined by measuring the content of nitrite, one of the end products of NO oxidation in the media, as previously described [16]. A standard nitrite curve (0.25–50 μM) was generated using a 10 mM solution of $NaNO_2$. All chemical for the NO assay were from Sigma (EU).

### 2.8. Arginase assay

The colorimetric assay for the measurement of arginase activity was performed in accordance to previously described protocols [17]. Briefly, cells were washed twice with PBS 0.5% BSA, then lysated at 37 °C for 25 min in 60 μl/well of lysis buffer (0.1% Triton X-100 in water, freshly completed with 10 mM leupeptin and 5 mM aprotinin) and subjected to 3 freeze-thaw cycles. Standard urea solutions (20 mM to 0.156 mM) were prepared by serial dilutions of 0.1 M urea in lysis buffer. Blank samples, consisting of lysis buffer without urea, were included. Following centrifugation at 240 rcf for 5 min, 50 μl of lysates, standard solutions and blanks were transferred into 1.5 ml microfuge tubes (remaining lysate aliquots are stored at −20 °C until protein quantification). 50 μl of 25 mM Tris–HCl and 10 μl of 50 mM $MnCl_2$ were added to all samples (lysates, standard solutions and blanks) and the mixture heated at 56 °C for 10 min. 100 μl of 500 mM L-arginine were added to all samples and incubated at 37 °C for 30 min. L-arginine hydrolysis was stopped by adding 800 μl of acid solution [96% $H_2SO_4$: 85% $H_3PO_4$: $H_2O$ (1:3:7)] to all samples. 40 μl of 9% α-isonitrosopropiophenone (in ethanol) was added to all samples and incubated for 45 min at 95 °C. At the end of incubation, 200 μl of each sample were transferred into 96-well flat-bottom plate and the absorbance read at 540 nm (reference filter 670 nm), using a microplate spectrophotometer reader. Arginase activity is given as μmoles/ml.

### 2.9. Determination of mitochondrial membrane potential

To measure mitochondrial membrane potential the potentiometric dye tetramethylrhodamine ethyl ester perchlorate (TMRE) was used at a final concentration of 30 nM (from 1 mM stock solution in DMSO). Cells were kept for 30 min in the presence of TMRE before recording, in order to reach saturation of the dye, and maintained throughout the entire experiment to avoid the decay of the signal. An oil immersion objective (Olympus: 40×, 1.35 NA) mounted on an inverted microscope (Axiovert 135, Zeiss; Germany) was utilized for fluorescence video imaging. The excitation wavelength 535 nm was applied by means of a monochromator (Till Photonics, Polychrome II; Germany) and the emission light at 590 was collected by a CCD, cooled digital camera (PCO, Sensicam; Germany) and recorded. The Imaging Workbench 6.0 software package (Indec BioSystems; CA, USA) was used for recording and off-line analysis of the data. The software allowed the measurement of the emission value in regions of interest or along line profiles, where peaks of amplitude corresponded to single mitochondria. The average amplitudes of the fluorescence intensity were calculated (from a minimum of 3 experiments for each condition) and shown in bar graphs as average ± SEM, with n = number of observations.

### 2.10. Western blot analysis

Cell lysates were prepared as previously described [16]. Briefly, microglial cells were lysed in ice cold RIPA buffer (50 mM Tris–HCl supplemented with 1% NP40, 0.1% SDS, 0.1% Sodium desoxycholate, 1X Protease Inhibitor Cocktail, Sigma, 10 mM NaF, 100 μM $Na_3VO_4$) and insoluble material removed by centrifugation (10,000 ×g at 4 °C, 10 min). Protein concentration was measured and equal amounts (50 μg) separated by 4–12% SDS-PAGE (Invitrogen) and transferred to nitrocellulose membranes. Membranes were blocked with 7% non-fat milk and incubated with rabbit anti-phospho-p70S6 Kinase (Cell Signaling; 1:1000), recognizing p70 S6 Kinase when phosphorylated at threonine 389, and rabbit anti-p70 S6 Kinase (Cell Signaling; 1:1000) antibodies, overnight at 4 °C. The housekeeping gene actin was recognized by mouse anti-actin antibody (Sigma; 1:5000, 1 h at RT). Horseradish peroxidase conjugated anti-rabbit and anti-mouse IgG (Thermo, Pierce 1:10,000, 1 h at RT) and enhanced chemiluminescence ECL reagents (Thermo, Pierce) were used as detection system. Band detection and image capture were performed by ChemiDoc XRS Densitometer (Bio-Rad) and Image LabTM 4.0 software; band quantification was performed by Image LabTM 4.0 software; intensities of bands corresponding to P-p70S6K and Tot-p70S6K were normalized over the corresponding actin band.

### 2.11. MTT crystal violet and protein assays

The ability of cells to reduce 3-(4,5-dimethyl thiazol-2-y1)-2,5-diphenyl tetrazolium bromide (MTT) was assessed as index of cellular

metabolic activity and mitochondrial integrity, as previously described [17]. MTT was added at a final concentration of 0.25 mg/ml during the final 4 h of incubation. The medium was then removed, and 100 μl DMSO added to each well to dissolve the dark blue crystals. The plates were then read on a microplate reader, using a test wavelength of 570 nm and a reference wavelength of 630 nm. The cell number in each condition was estimated by crystal violet (CV) dye [18]. Protein concentration was measured by BCA protein assay (Thermo, Pierce).

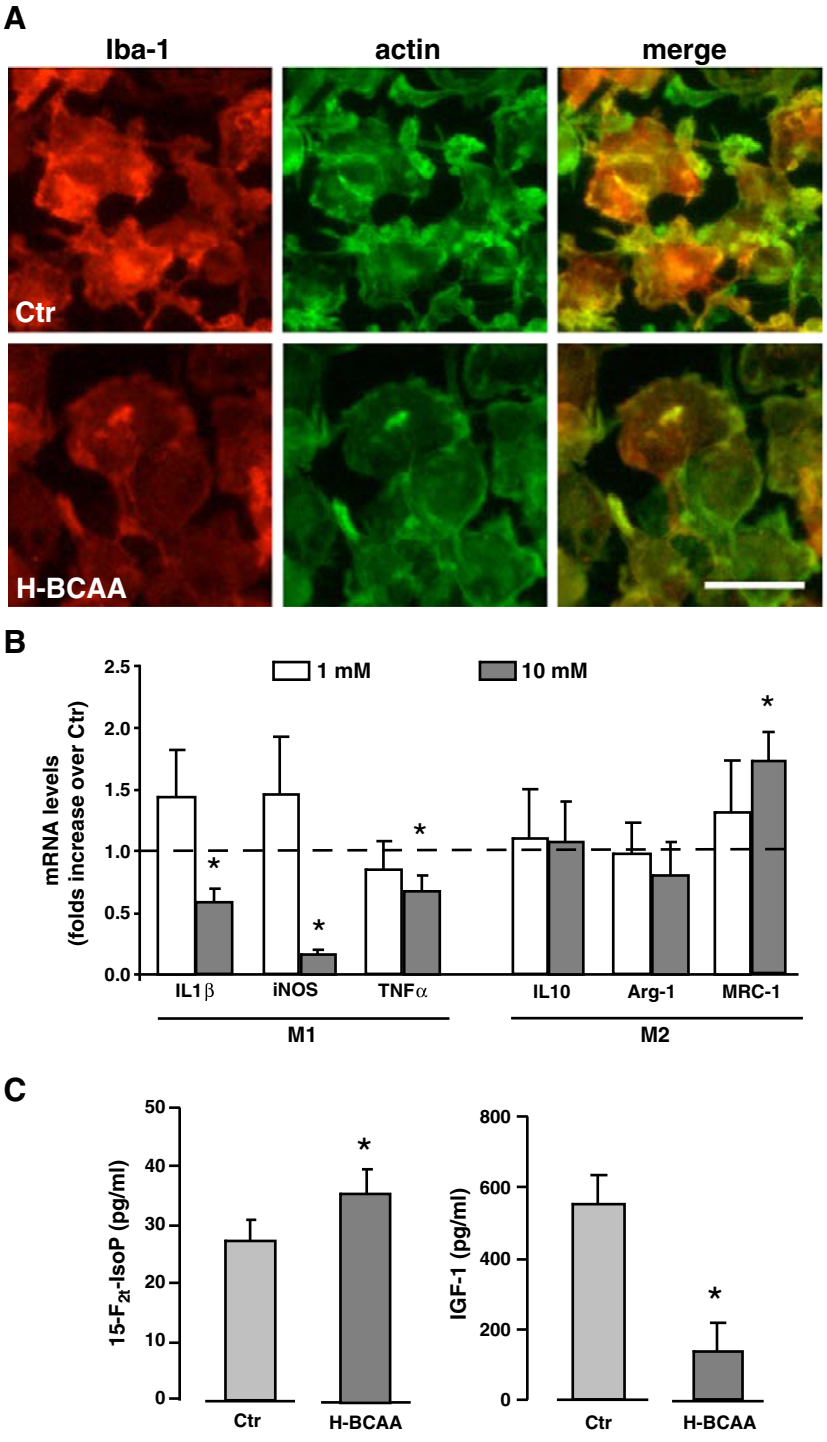


**Fig. 1.** Effects of high levels of BCAAs on microglia morphology, gene expression, free radical generation and IGF-1 production. Microglial cells, harvested from mixed glial cultures that had been cultivated in normal (Ctr) or high BCAA medium (H-BCAA), were maintained for a further 24 h in the two culture conditions (Ctr and H-BCAA) and then processed for the indicated analysis. A. Photomicrographs showing the morphology of microglial cells grown in control (Ctr) or in 10 mM BCAA supplemented medium (H-BCAA). Cells were immunostained for the microglial marker Iba-1 (red) followed by phallacidin staining (green) to selectively label filamentous actin. Scale bar = 25 μm. B. mRNA analysis by real time PCR of M1 and M2 genes in H-BCAA (1 and 10 mM) microglial cultures. Data are given as fold change over the expression of the corresponding gene in Ctr cultures taken as 1 (dotted line). * $p<0.05$ versus Ctr cultures. Data are mean ± SEM of 3 to 5 independent experiments, run in duplicates. C. Left panel: accumulation (24 h) of the lipid peroxidation product 15-$F_{2t}$-IsoP, as index of free radical generation, in the culture media of Ctr and H-BCAA cultures. * $p<0.01$ versus Ctr cultures. Data are mean ± SEM of 6 independent experiments, run in duplicate. Right panel: Accumulation (24 h) of IGF-1 in the culture media of Ctr and H-BCAA cultures. * $p<0.01$ versus Ctr cultures. Data are mean ± SEM of 3 independent experiments, run in duplicate.

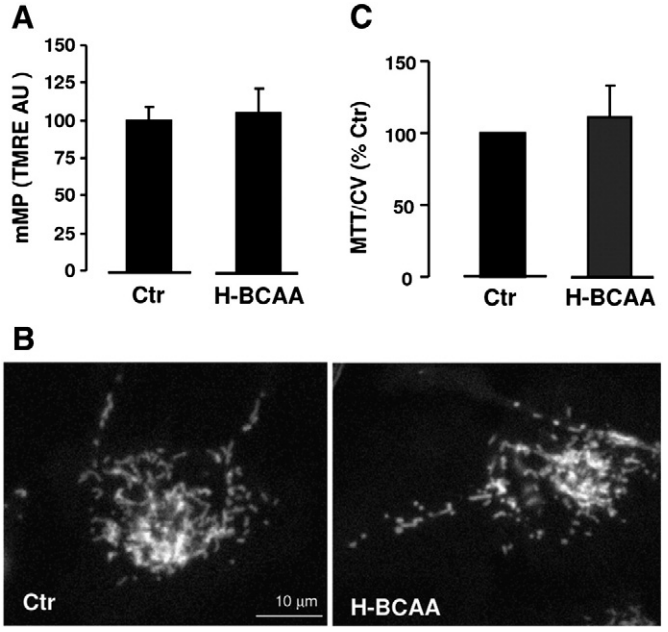


**Fig. 2.** Effects of high levels of BCAAs on mitochondrial function and morphology. Cells were harvested and maintained as for Fig. 1. A. Mitochondrial membrane potential (mMP) in the two culture conditions was measured by using the potentiometric dye TMRE in single cell video-imaging experiments. Data are mean ± SEM and are expressed as arbitrary units (AU). B. Example of mitochondrial shape and density in microglial from Ctr and H-BCAA cultures. C. Mitochondrial activity was also measured by MTT reduction; the values of MTT reduction were normalized for the cell number, evaluated by CV assay. Data are given as mean ± SEM of MTT/CV, expressed as % of control; n = 5 independent experiments, run at least in duplicate.

## 3. Statistical analysis

Data are expressed as means + SEM of (n) independent experiments (run in duplicate). Statistical significance was evaluated using Student's t test or one-way ANOVA followed by Bonferroni's correction for multiple comparisons. Numbers of independent experiments are indicated in the figure legends; $p<0.05$ was accepted as statistical significance. Analyses were performed using StataTm 8.1 software (Stata Corporation, College Station, TX).

## 4. Results

### *4.1. High levels of BCAAs influence microglial gene expression profile and free radical production*

Microglial cells were harvested from mixed glial cultures that had been cultivated in control (Ctr) or high BCAA medium (H-BCAA) for

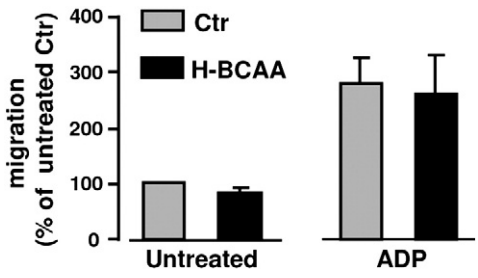


**Fig. 3.** Effects of high levels of BCAAs on microglial motility and migration. Motility of microglial cells was assessed using transwell migration chambers. Microglial cells from Ctr and H-BCAA cultures were plated into the top wells ($6\times10^4$ cells/well) in control medium or H-BCAA, without serum; bottom wells were filled with medium alone or medium containing 10 μM ADP. Cells were incubated for 4 h at 37 °C and after fixation and H&E staining, 16 fields were examined for each condition. Data are given as % of cells migrated in untreated control cultures, taken as 100%; n = 3 independent experiments.

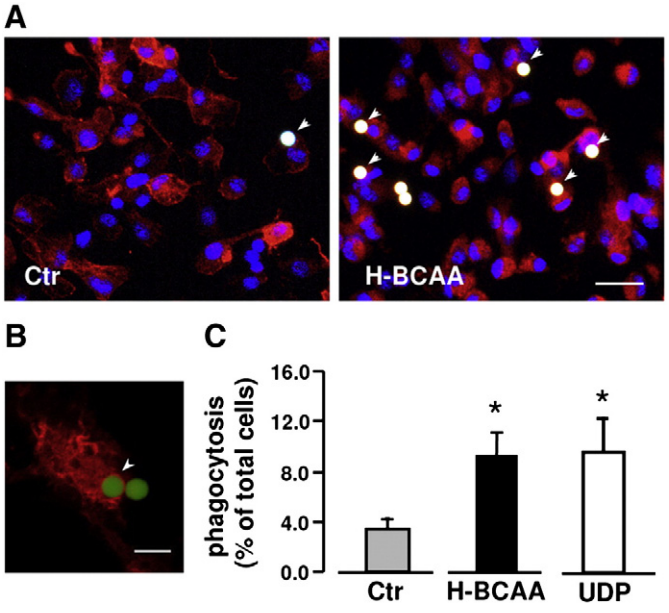


**Fig. 4.** Effects of high levels of BCAAs on microglial phagocytosis of opsonised fluorescent beads. Microglial cells from Ctr and H-BCAA cultures were incubated with 6 μm diameter beads at 37 °C for 45 min. As positive control, phagocytic activity was induced by pre-incubating microglial cells with 100 μM UDP for 30 min at 37 °C before adding the beads. At the end of incubation, cells were washed twice, fixed and processed for Iba-1 staining. Phagocytosis was examined by fluorescence microscopy (A). Arrow heads in panel A indicate engulfed beads (scale bar = 25 μm). In panel B, a confocal microscopy image of particles engulfing a cell (arrow head) is given: scale bar = 10 μm. The percentage of Iba-1 positive cells engulfing fluorescent beads on total cell number in each microscopic field (10 fields for each condition) was counted and given as mean ± SEM (n = 3 independent experiments, run in triplicates). *$p<0.05$ versus control.

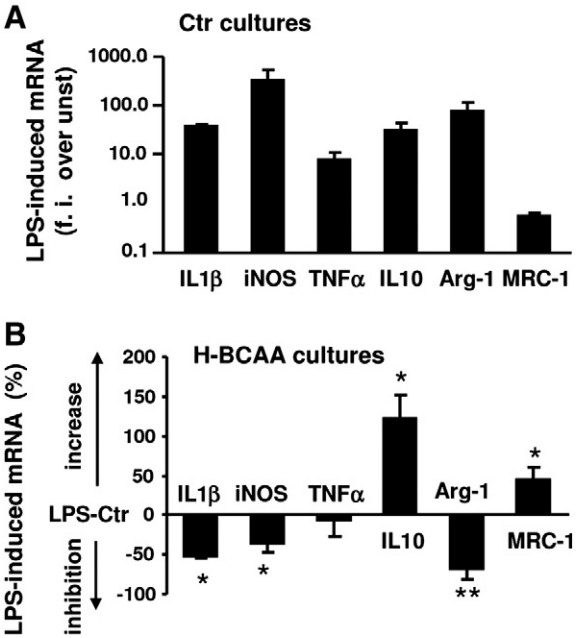


**Fig. 5.** Effects of high levels of BCAAs on LPS-induced M1 and M2 gene expression. A. mRNA analysis by real time PCR of M1 and M2 genes induced by LPS in Ctr microglial cultures. Cells were stimulated with 10 ng/ml LPS for 6 hrs, harvested and cDNA analysed by real time PCR. Data are given as folds increase over the expression of the corresponding gene in unstimulated Ctr cultures. Data are mean ± SEM of 3–5 independent experiments, run in duplicate. All values are significantly different from control. B. mRNA analysis by real time PCR of M1 and M2 genes induced by LPS in H-BCAA cultures. Cells were treated and analysed as described for panel A. Data are given as % of increase or decrease over the corresponding gene in LPS-control medium (Ctr) cultures, with positive values indicating an increase in the expression of the gene in H-BCAA cultures and negative values an inhibition. Data are mean ± SEM of 3–5 independent experiments, run in duplicate. *$p<0.05$ and **$<0.001$ versus LPS-stimulated Ctr cultures.

6 days. After isolation, microglial cells were maintained for further 24 h in the two culture conditions (Ctr and H-BCAA) and then processed for the indicated analyses. The concentrations of BCAAs (1 and 10 mM), were selected on the basis of previously reported in vitro studies [9,19] to mimic the abnormal BCAA plasma levels reported in MSUD patients or in athletes after acute administration of BCAAs as dietary integrators to improve physical performance and fatigue resistance [2]. At 10 mM concentration, BCAAs did not affect microglial viability (see material and method section) or total protein synthesis ($40 \pm 6$ and $51 \pm 8$ μg/$10^5$ cells, for Ctr and H-BCAA cultures, respectively; $n = 6$). Cell morphology (Fig. 1A), was modestly affected by H-BCAAs as assessed by staining microglial cultures with the cytosolic $Ca^{2+}$ binding protein Iba-1, although staining for F-actin revealed some differences suggestive of cytoskeleton reorganization. Doubly stained cells (Fig. 1A, right panels), showed a localisation of both Iba1 and F-actin at the rim of the cell membranes, consistent with the ability of Iba-1 to bind actin and with its involvement in RacGTPase-dependent membrane ruffling and phagocytosis [20,21].

The analysis of the expression of a panel of genes associated either to M1 (IL-1β, iNOS, TNF-α), or M2 (IL-10; arginase-1, Arg-1; mannose receptor, MRC-1), microglial phenotype (Fig. 1B), revealed lower levels of transcripts encoding for all three M1 genes in microglial cultures exposed to 10 mM BCAAs as compared to control cultures. Of the typical M2 genes examined, MRC-1 expression was significantly increased in H-BCAA cultures while the expression of IL-10 and Arg-1 remained unaffected. mRNA levels of all six genes were not significantly altered in cultures exposed to 1 mM BCAAs. Thus, we focused most of our further analyses on cultures exposed to 10 mM BCAAs.

Next, we analysed the accumulation in the culture media of 15-$F_{2t}$-IsoP, a lipid peroxidation product and a well established oxidative stress biomarker [22], and of IGF-1, an important microglial product with neuroprotective and anti-inflammatory activities. We found a moderate, albeit significant, increase in the 15-$F_{2t}$-IsoP levels (Fig. 1C, left panel), and a strong reduction in IGF-1 production (Fig. 1C, right panel) in H-BCAA cultures as compared to control cultures.

These results suggest that BCAAs, either indirectly (through their potential actions on astrocytes during the 6 days in the mixed glial cultures), or directly (during the 24 h incubation of purified microglial cultures), influence the functional properties of microglial cells and induce an incomplete polarisation toward the M2 phenotype.

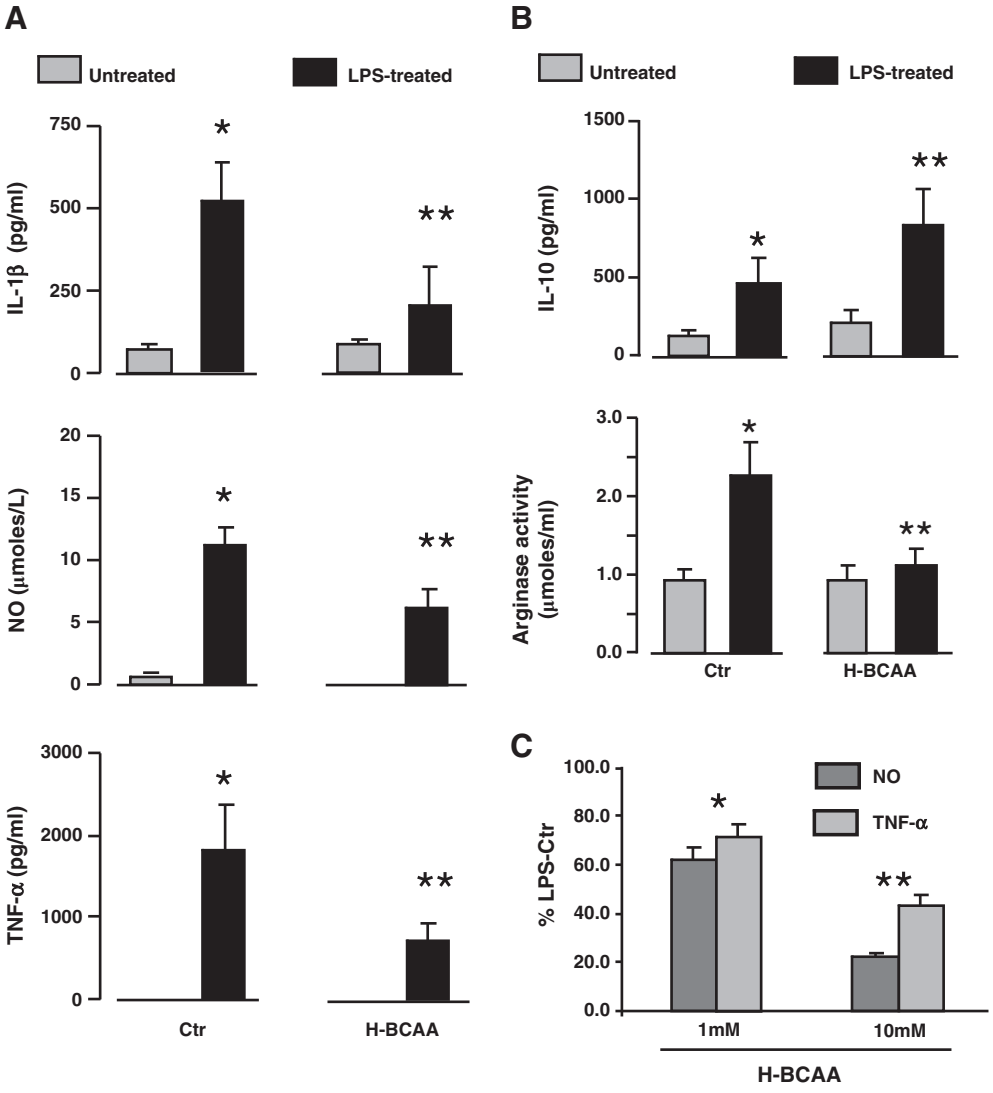


**Fig. 6.** Effects of high levels of BCAAs on LPS-induced cytokine and NO production and arginase activity. Microglial cells from Ctr and H-BCAA cultures were incubated for 24 h in the absence (untreated) and in the presence of 10 ng/ml LPS (LPS-treated). Accumulation of IL-1β, nitrite, TNF-α (panel A) and of IL-10 (B) in the culture media was then quantified as detailed under Materials and methods section. Arginase activity is also shown in panel B. *$p<0.05$ versus Ctr cultures, ** $p<0.05$ versus LPS-stimulated Ctr cultures. Accumulation of nitrite and TNF-α in cells maintained in culture medium supplemented with 1 mM BCAA and stimulated by LPS are given in panel C. Data are expressed as % of metabolite levels induced by LPS in Ctr cultures. *$p<0.05$ versus Ctr cultures, ** $p<0.05$ versus Ctr cultures and 1 mM BCAA cultures. Data are mean ± SEM of $n = 5$ independent experiments, run in duplicate.

### 4.2. High levels of BCAAs do not influence mitochondrial membrane potential and morphology

Since BCAAs have been shown to impair mitochondrial functions possibly through the generation of free radicals at mitochondrial level [23], we measured the mitochondrial membrane potential (mMP) as index of electron transport chain integrity. Changes in mMP ($\Delta\psi m$) were measured by using the potentiometric dye TMRE in single cell video-imaging experiments. The intensities of the TMRE fluorescence signal were comparable in the two culture conditions (Ctr and H-BCAA; Fig. 2A), thus suggesting that BCAAs do not induce mitochondrial membrane depolarization. The two cell culture types also showed similar MTT reducing activities (Fig. 2B); MTT is taken up by living cells and it is reduced by mitochondrial NADH-dependent dehydrogenases (mainly succinate dehydrogenase or complex II of the respiratory chain), thus providing a measure of the mitochondrial activity, generally used as an index of cell viability. The absence of mitochondrial damage, suggested by the mMP and MTT measurements, was consistent with the preserved rod-like shape morphology of mitochondria in both Ctr and H-BCAA cultures, as evidenced by visual inspection of TMRE loaded mitochondria (Fig. 2C).

### 4.3. High levels of BCAAs do not affect microglial motility but promote phagocytosis

We next analysed whether the altered gene expression induced by BCAAs could influence important functions such as motility and phagocytosis. We performed a chemotaxis assay to evaluate the basal motility of control and H-BCAA microglial cells as well as their ability to respond to a well defined microglial chemoattractant such as ADP [15]. As shown in Fig. 3, there was no difference between Ctr and H-BCAA cultures, in both basal and ADP-induced microglial motility.

To examine phagocytosis, we assessed the engulfment of opsonised fluorescent latex beads by Iba-1 positive cells, a specific marker for microglia (Fig. 4). We observed a higher percentage of particle-engulfing cells in H-BCAA cultures than in Ctr cultures. Phagocytosis in H-BCAA microglia was comparable to that evoked by 100 μM uridine 5 -diphosphate (UDP), a known inducer of microglial phagocytosis [24].

### 4.4. High levels of BCAAs alter microglial immunological profile in response to LPS activation

Finally, we investigated the expression profile of M1 and M2 genes in response to a prototypical inflammatory stimulus such as LPS. As expected, in control cultures, LPS decreased MRC-1 gene expression and increased the levels of the transcripts for the remaining genes (Fig. 5A). When the LPS-induced mRNA levels in H-BCAA cultures were compared to those induced in control cultures, we observed a reduced ability of H-BCAA microglia to express IL-1β and iNOS (Fig. 5B). The decreased mRNA levels were reflected in decreased amounts of IL-1β and NO (measured as nitrites) accumulated in culture media (Fig. 6A). A significant decrease in IL-1β and nitrite levels was also observed in cultures maintained in 1 mM BCAA-supplemented medium and challenged with LPS (Fig. 6C). In the case of TNF-α, there was no difference in mRNA levels between Ctr and H-BCAA microglial cells but at protein levels TNF-α was substantially reduced in H-BCAA cultures, indicating a post-transcriptional regulation of this gene by BCAAs (Figs. 5B and 6A, bottom panel).

Among the M2 genes, IL-10 and MRC-1 mRNA levels were increased (Fig. 5B). The increased expression of IL-10 in H-BCAA cultures was confirmed at protein level (Fig. 6B). However, when looking at Arg-1, a

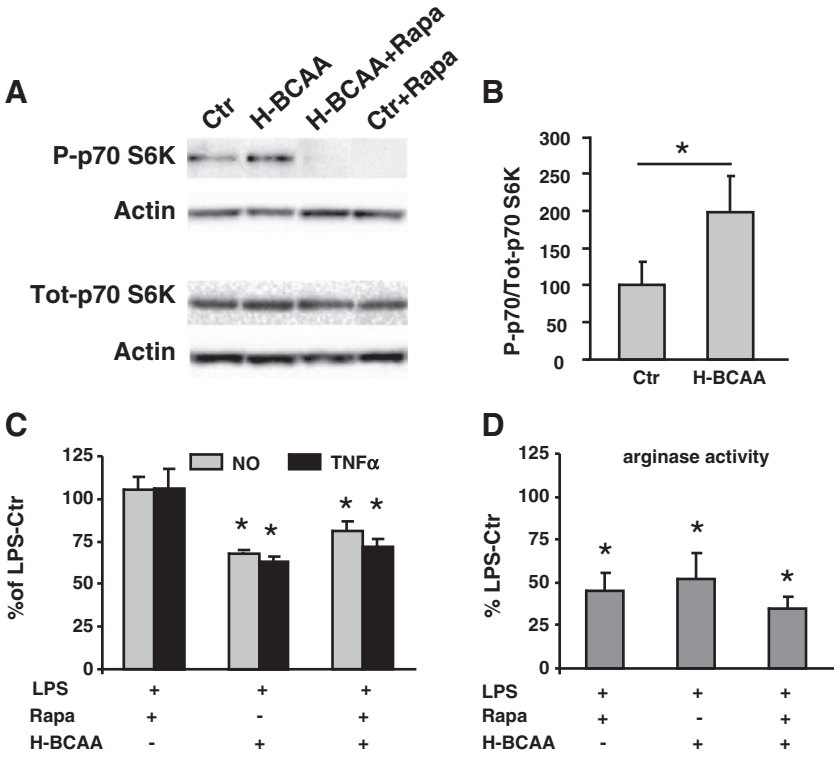


**Fig. 7.** Effects of rapamycin and high levels of BCAAs on LPS-induced NO, TNFα and arginase activity. A. Phosphorylated p70 6S Kinase and total p70 6S kinase levels in Ctr cultures and H-BCAA cultures, with and without 5 nM rapamycin. Microglial proteins were analyzed by western blot analysis. Actin was used as housekeeping gene. Data representative of n = 3 independent experiments, run in duplicate. B. Ratios of phosphorylated p70 S6K (P-p70) and total p70 S6K (Tot-p70) in control and H-BCAA cultures, after actin normalization; data are expressed as % of control and are mean ± SEM of n = 3 independent experiments, run in duplicate; *p < 0.05. C. Microglial cells from Ctr and H-BCAA cultures were stimulated for 24 h with LPS (10 ng/ml) in the presence of 5 nM rapamycin. Accumulation of nitrite and TNF-α were then determined. Results are given as % of LPS-induced metabolite or activity in Ctr cultures. *p < 0.05 versus LPS-stimulated Ctr cultures. D. Arginase activity was measured in microglial cultures as described in (C). Results are given as % of LPS-induced metabolite or activity in Ctr cultures. *p < 0.05 versus LPS-stimulated Ctr cultures. Data in panels C and D are mean ± SEM of n = 5 independent experiments, run in duplicate.

gene whose expression is increased in M2 as compared to M1 macrophages, both mRNA expression and enzymatic activity were substantially lower in H-BCAA than in Ctr microglial cells (Figs. 5B and 6B).

### *4.5. Rapamycin does not prevent the altered response to LPS of microglial cells exposed to high levels of BCAAs*

It has been reported that many of the activities of BCAAs are mediated by signalling involving the mammalian target of rapamycin (mTOR). We first evaluated the phosphorylation of p70 S6 Kinase, a Ser/Thr kinase downstream to mTOR activation (Fig. 7A–B). We used an antibody recognizing p70 S6 kinase upon phosphorylation of Thr389, which closely correlates with in vivo p70 S6 kinase activity and is blocked by rapamycin. The level of phosphorylated p70 6S Kinase was increased in H-BCAA cultures as compared to Ctr cultures. Such phosphorylation was abrogated in the presence of rapamycin, used at 5 nM concentration according to previous studies on primary microglial cultures [25,26]. In H-BCAA cultures, rapamycin did not affect cell viability or number (MTT assay, $107 \pm 14$ and CV assay, $103 \pm 3\%$ of control for control). In control cultures, the presence of rapamycin did not affect cell viability ($91 \pm 8\%$ of control cultures, $n = 5$) but slightly decreased the cell number ($92 \pm 1\%$ of control, $p < 0.05$, $n = 5$).

The presence of 5 nM rapamycin did not influence the levels of nitrite or TNF-α induced by LPS and it did not reverse the inhibitory effects exerted by high levels of BCAAs (Fig. 7C). We also tested the effect of rapamycin on LPS-induced arginase activity (Fig. 7D). In control cultures, rapamycin significantly reduced arginase activity, suggesting that mTOR signalling is involved in Arg-1 expression following LPS-activation, as described for other M2 genes in monocytes and dendritic cells [27]. Rapamycin had no effect on arginase activity in H-BCAA cultures exposed to LPS, nor on basal levels of nitrite, TNF-α and arginase (not shown). Altogether, these data indicate that inhibition of mTOR by rapamycin does not counteract the effects of BCAAs, at least as far as NO and TNF-α production and arginase activity is concerned.

## 5. Discussion

BCAAs are important nutrients and signalling molecules, acquired with diet, with critical roles for normal growth and functions of the whole organism. During exercise or starvation, protein breakdown increases BCAA availability and catabolism, which occurs largely in mitochondria of brain, muscles and adipose tissue [3]. The final products of BCAA catabolism then enter the tri-carboxylic acid cycle to produce reduced nicotinamide adenine dinucleotide for mitochondrial respiration. In addition, within the brain, BCAAs participate either directly or indirectly to the synthesis of neurotransmitters and in maintaining the nitrogen balance of the glutamate–glutamine cycle between astrocytes and neurons [28,29]. BCAA homeostasis is therefore essential to normal brain physiology.

In the present study, we show that the protracted exposure to high levels of BCAAs alters the immune properties of microglia, the main immunocompetent cells of the brain parenchyma, and suggest a novel action for BCAAs as modulators of central immune response.

Microglial cells cultured in high BCAA-containing medium under unstimulated conditions exhibit a lower expression of the M1 genes IL-1β, TNF-α and iNOS and a concomitant higher expression of the M2 gene MRC-1. These features would suggest a less reactive phenotype. However, these cells are also characterized by an increased production of reactive oxygen species, as indicated by the higher levels of the lipid peroxidation product 15-$F_{2t}$-IsoP, and by a lower production of the neuroprotective factor IGF-1, suggesting an unbalance in favour of neurotoxic activities.

H-BCAA microglial cultures show an increased capacity to phagocyte opsonised fluorescent latex beads, in agreement with the enhanced levels of transcripts for MRC-1 (or CD206), one of the several receptors mediating phagocytosis in microglial and perivascular macrophages [30]. Phagocytosis is an important physiological function of microglia, as clearance of dead cells or dangerous debris is crucial to the maintenance of brain functions and sustained phagocytic activity is typical for M2 macrophages and microglia [31,32].

Other microglial functions, such as basal motility and migration in response to a well defined microglial chemoattractant such as ADP, were comparable in control and H-BCAA cultures.

Furthermore, although mitochondrial respiratory chain activity was decreased in rat cerebral cortex slices acutely exposed to mM concentrations of BCAAs [23], H-BCAA microglial cultures presented unaltered mitochondrial functions, as indicated by morphology and measurements of mMP and MTT reduction activity. In line with our observations, 10–50 mM BCAAs did not induce mitochondrial membrane depolarisation in glial and neuronal cell lines [19]. Mitochondrial respiratory chain dysfunctions are known to hamper the process of microglial alternative activation [33], but our observations suggest that mitochondrial impairment is not the mechanism by which BCAAs prevent the acquisition of a full M2 phenotype.

A further result of our study is that H-BCAA microglial cells respond differently to Toll receptor-4 (TLR-4)-mediated activation as compared to control microglial cultures. In H-BCAA cultures, the levels of NO, IL-1β and TNF-α induced by LPS were significantly decreased, suggesting a milder inflammatory reaction. Other M2-associated genes, such as IL-10 and MRC-1, were increased, suggesting that, in the presence of high BCAA levels, the pattern of activation is skewed towards the M2 phenotype. Nonetheless, both Arg-1 mRNA expression and arginase activity were substantially lower than in Ctr cultures, further supporting the failure of H-BCAA cultures in acquiring a full M2 phenotype. Arginase-1 competes with iNOS for the common substrate L-arginine and transforms it into urea and ornithine, the precursor of a family of small molecules termed the polyamines. Spermidine and spermine, two of the most important polyamines, have neuroprotective functions and are essential for neurite outgrowth, neuronal survival and regeneration [34,35]. As for the decreased synthesis of IGF-1 in unstimulated cultures, the low levels of expression/activity of Arg-1 in activated H-BCAA microglia could result in a substantial lack of neurotrophic activities.

Among the molecular regulators of innate immune response is mTOR pathway. In LPS-stimulated human monocytes and dendritic cells, mTOR activity has been suggested to regulate M1/M2 gene expression by favouring M2 phenotype [27]. However, in LPS-stimulated PBMC [36] and LPS-activated microglial cells [25, and the present study], mTOR seems to fulfil this role only as far as M2 genes is concerned since iNOS/NO and TNF-α levels were not affected by rapamycin whereas arginase activity was substantially inhibited. Many of the activities of BCAAs are mediated by mTOR, including the recently described hyperexcitability of cortical neurons [10]. Under our experimental conditions rapamycin did not prevent the BCAA-dependent decrease in NO and TNF-α production induced by LPS. Furthermore, both rapamycin and BCAAs lowered arginase activity and the results were not additive. These observations suggest that mTOR is not necessary for BCAA activity in LPS-activated microglial cells.

Altogether, our observations suggest that the exposure to high levels of BCAAs affects the microglial immune properties in a peculiar manner, promoting the acquisition of an intermediate phenotype that is less inflammatory than the M1 and less protective than the M2. We hypothesize that this unique functional phenotype could result in a low-grade inflammatory state and a less efficient microglial response to local damage, two features that might increase the susceptibility to neurodegenerative processes. In this light, loss of microglial surveillance function has been recently described in a mouse model of amyotrophic lateral sclerosis, during the clinical phase of disease [37]. These authors suggest that the altered functions of microglia may be instrumental for neurodegeneration: if on one side, an overactive reaction to tissue

damage may be harmful to neurons and axons, on the other a rapid response is required for an efficient clearance of debris and neurotoxic molecules, and the resolution of the ongoing focal neurodegeneration.

Although based on in vitro evidence, our results add on to an increasing literature casting doubts on the safety of large consumption of BCAAs as dietary integrators. BCAA dietary supplementation is indicated in pathologic conditions in which plasma BCAA concentration is significantly reduced – e.g. hepatic cirrhosis, traumatic brain injury, obesity and geriatric patients [38] – with the intention of normalizing amino acid profiles and nutritional status. The mechanisms by which BCAAs exert beneficial effects in these pathological states are not fully elucidated but they vary, according to the specific tissue target (adipose tissue, muscles, brain). In addition, BCAA supplementation is commonly used to improve physical and mental performance in athletes [2,4,39]. Depletion of BCAAs in muscle and plasma induced by exercise can produce a negative impact on muscle energy economy. Since BCAAs enter the brain through a transporter that they share with a number of large neutral amino acids including tryptophan, a drop in BCAA levels could indirectly increase tryptophan uptake into brain and stimulate neuronal synthesis and release of serotonin. On this basis, the assumption that high BCAA intake could prevent muscle and "central" fatigue has been put forward although no conclusive results on the beneficial effects of BCAA supplementation in athletes are yet available [2]. In contrast, there is an increasing concern on the abuse of BCAA-containing dietary integrators and energetic drinks, particularly in adolescents and young adults [40–42]. In addition to a decrease in serotonin synthesis and release, a high intake of BCAAs could result in increased levels of glutamate, formed from BCAA transamination. Glutamate is the main excitatory neurotransmitter and the substrate for the synthesis of the major inhibitory neurotransmitter γ-aminobutyric acid (GABA). It has been proposed that BCAAs and their branched-chain α-keto-acids participate in maintaining the nitrogen balance in the glutamate-glutamine cycle between astrocytes and neurons, serving as "shuttle" molecules for amino groups [28,29,43]. An excess of BCAAs could upset the balance and increase the likelihood of excitotoxicity. In this context, it is tempting to speculate that a less efficient microglial surveillance could impede damage resolution and allow a condition of detrimental low-grade inflammation.

Of note, on the basis of epidemiological and experimental studies, the excessive use of dietary integrators including BCAAs has been proposed as one of the factors that could explain the increased risk of amyotrophic lateral sclerosis in professional soccer players as compared to the general population [44–47].

## 6. Conclusions

This study shows that in the presence of high levels of BCAAs microglial cells exhibit a peculiar phenotype characterized by a partial skewing toward the M2 state that could results in a less efficient microglial response to local damage and the establishment of a low-grade inflammatory state. In addition to the described BCAA-induced hyperexcitability and excitotoxiticy, the altered immune profile of microglia may represent a further mechanism by which important nutrients and potent signalling molecules such as BCAAs might turn into toxicants and increase the likelihood of neurodegenerative events.

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